CHLOROPLAST INCLUSIONS IN *BONNEMAISONIA HAMIFERA* (RHODOPHYTA, BONNEMAISONIALES)

Curt M. PUESCHEL and Susan J. BABUKA

Department of Biological Sciences, State University of New York at Binghamton, Binghamton, NY 13902-6000, USA

ABSTRACT - Transmission electron microscopy revealed the presence of electron-dense inclusions in chloroplasts of the tetrasporophytic *Trailliella* phase of *Bonnemaisonia hamifera* (Hariot) Okamura. Each inclusion was composed of expansive sheets, 3.5-4.0 nm thick, that were fully appressed and formed stacks of as many ▬ fifteen layers. Stacks occurred singly or in aggregations whose members had divergent and sometimes radial orientation. Some stacks were embedded in an amorphous electron dense matrix. The inclusions were located among the thylakoids in the stroma. Even in unstained sections, the inclusions were electron-dense, suggesting that the material was either strongly osmiophilic or natively electron-dense. X-ray microanalysis of the inclusions did not reveal any element, other than osmium introduced during postfixation, that was unusual or that would confer electron density. The electron density of the inclusions must be the result of an osmiophilic nature.

RÉSUMÉ - La microscopie électronique à transmission ▪ révélé des inclusions denses aux électrons dans les plastes de *Trailliella*, tétrasporophyte de *Bonnemaisonia hamifera* (Hariot) Okamura. Chaque inclusion est composée de feuillets expansifs de 3.5-4.0 nm d'épaisseur, complètement apprimés et formant des empilements jusqu'à quinze couches. Les empilements sont isolés ou forment des agrégats dont les membres adoptent ▬ orientation divergente, parfois radiale. Quelques empilements se trouvent dans une matrice amorphe dense aux électrons. Ces inclusions sont situées parmi les thylacoïdes dans le stroma. Même sans agent de contraste, elles sont denses aux électrons, ce qui suggère que le matériel est soit fortement osmiophile soit d'une nature dense aux électrons. Leur microanalyse aux rayons X ne révèle pas d'élément, autre que l'osmium provenant de la postfixation, qui soit inhabituel ou qui conférerait l'opacité aux électrons. La densité électro-nique des inclusions doit être la conséquence de la nature osmiophile du matériel.

KEY WORDS - chloroplast, *Bonnemaisonia hamifera*, ultrastructure.

INTRODUCTION

Chloroplasts of red algae have been reported to contain a number of unusual inclusions (Pueschel, 1990). With the exception of aggregations of the iron-containing protein, ferritin (Pueschel & Cole, 1980; Pueschel & Parthasarathy, 1984), the composition and possible physiological roles of these inclusions are unknown. Cytoplasmic protein crystals are widespread in red algae (Pueschel, 1992), but

chloroplast crystals are not. Chloroplast inclusions in *Asterocolax gardneri* (Setch.) J. & G. Feldmann had crystalline substructure but not a crystalline outline (Goff, 1982); those in *Antithamnion defectum* Kylin had a polyhedral outline, but no crystalline substructure was demonstrated (Young, 1979). Chloroplast inclusions, also presumably proteinaceous but irregularly shaped and without apparent substructure, were found in *Palmaria palmata* (L.) O. Kuntze (Pueschel & van der Meer, 1984), whereas amorphous masses of granular material were present in plastids of *Hildenbrandia rubra* (Sommerf.) Menegh. (Pueschel, 1988).

In the course of ultrastructural examination of the *Trailliella* phase of *Bonnemaisonia hamifera* (Hariot) Okamura, chloroplast inclusions were encountered that were unlike any inclusion previously reported to occur in either chloroplasts or cytoplasm of red algae. Their structure is described in the present report.

MATERIAL AND METHODS

Cultures of the *Trailliella* phase of *Bonnemaisonia hamifera* (as *Trailliella intricata*) were obtained from Connecticut Valley Biological Supply and the University of Texas Culture Collection of Algae. Specimens from cultures of different ages were fixed according to a variety of protocols: 3% glutaraldehyde, 29% formaldehyde, 0.2 M sucrose in 0.1 M sodium cacodylate buffer, 5% glutaraldehyde, 0.2 M sucrose in 0.1 M sodium cacodylate buffer, or 2.5% glutaraldehyde, 0.5 caffeine in 0.05 M phosphate buffer; all at pH 7.0 for 2 hours. After dilution and rinses with pure buffer, specimens were fixed in 1-2% aqueous osmium tetroxide for 1-3 hours, then rinsed with water, dehydrated in acetone, and embedded in Epon or Spurr's medium. Sections were stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate or were examined without staining.

Energy dispersive X-ray microanalysis was performed using a PGT System 4 interfaced with a Hitachi H-7000 TEM. Spectra were collected from unstained sections for 100 seconds at 75 kV with the microscope in scanning-transmission mode.

OBSERVATIONS

Chloroplasts in mature vegetative cells of the *Trailliella* phase of *Bonnemaisonia hamifera* were found to have unusual electron-dense inclusion within the stroma (Figs 1-4). The inclusions were not common, but they were present in all fixation regimes, which also represented different cultures ages. The abundance of chloroplast inclusions varied among cells of ■ single thallus. Some cells had several inclusions visible in a single section (Fig. 1); other cells had none.

The stroma around some inclusions was free of thylakoids, but commonly thylakoids were found to interdigitate with and align to projecting surfaces of inclusions (Fig. 1-3). Some plastoglobuli were unusually large and unevenly electron-dense (Figs 1, 2, 4), presumably due to partial extraction of lipids during fixation. Another unusual feature noted was extensive vesiculation of the chloroplast envelope

Fig. 1 - Thin-section electron micrograph of *Trailliella* phase of *Bonnemaisonia hamifera*. Electron-dense chloroplast inclusions are composed of a few elements or a cluster of radiating elements. Stroma near the large inclusion contains thylakoids, but one thylakoid (arrow) is interdigitated with the inclusion. Plastoglobuli (P) close to the inclusion ■ large and some are partially extracted. Chloroplast envelopes are highly vesiculated.

(Figs 1-3). In contrast, mitochondrial membranes, thylakoids, and other cellular membranes did not exhibit this behavior (Fig. 1).

The fundamental structural unit of the chloroplast inclusion was a flat sheet (Figs 1-5). Some sheets occurred singly, but most were assembled to form stacks. The surfaces of sheets in a stack were fully appressed. As many as 15 sheets were found in a single stack. In lateral view, a sectioned stack was found to be composed of alternating electron-dense and electron-transparent layers (Fig. 5). The thickness of a single sheet within a stack was approximately 3.5-4.0 nm. The electron-dense portion of the sheet was about half of the total thickness. It could not be determined whether the electron-

Fig. 2, 3 - Chloroplasts of *Trailliella* phase of *Bonnemaisonia hamifera*. The chloroplast inclusions consist of numerous radiating elements. Thylakoids (arrows), some of which are in oblique view, are aligned parallel to some of the surfaces of the inclusions. Note the proximity and similarity of small thylakoids and small sheets of inclusion material (arrowheads) in Fig. 2.

dense layer of a single sheet was covered on each side by a 1.0 nm thick electron-transparent layer, or on only one side by ■ 2.0 nm thick layer. Both configurations could produce the images observed. Oblique and face views of the sheets revealed no visible substructure. In these orientations, the thin sheets could easily be mistaken for amorphous inclusions.

Sheets or stacks of sheets were observed to occur alone or in aggregations (Fig. 1). Clusters of stacks often appeared to radiate from ■ central point (Figs 1 and 3). Some such aggregations appeared to be embedded in an amorphous electron-dense matrix (Fig. 4). The largest aggregation observed was about 2 μm in diameter.

To evaluate the source of the electron density of the chloroplast inclusions, some sections were examined without uranyl acetate or lead citrate staining (Fig. 5). The contrast of the inclusions was reduced, but they were still electron-dense. The layered substructure of the elements of a stack was more easily seen without section staining.

Energy dispersive X-ray microanalysis of the inclusions and of the surrounding stroma did not reveal the presence of unusual elements, except osmium, and this was expected to be present due to its incorporation during postfixation. The electron-density of the inclusions is apparently the result of the ability to bind osmium.

DISCUSSION

The layered structure of the chloroplast inclusions in *Bonnemaisonia* is somewhat suggestive of ■ membranous constitution. Tightly coiled membranes have been reported to occur in chloroplasts, especially developing chloroplasts, of several red algae (Delivopoulos & Kugrens, 1985). However, the thickness and electron-density of the inclusions in *Bonnemaisonia* are clearly different from normal membranes, and the elements are not whorled or coiled; they are stack units that terminate in free ends.

The possibility that these inclusions are artifacts created during processing or normal constituents altered from their native state was considered. However, spacing of thylakoids is normal, thylakoids interdigitate with the inclusions, and the density of granular stromal constituents close to the inclusions is the same as elsewhere in the chloroplast. The occurrence of inclusions under a variety of fixation conditions also argues against the possibility that these are artifacts.

The alignment of thylakoids parallel to surfaces of inclusions raises the question of whether there is perhaps a developmental relationship between the two. The similarity of size and disposition of thylakoids and sheets of inclusion material, as in figure 2, are particularly suggestive of such an ontogenic relationship. The formation of these inclusions might result from an unusual concentration of normal membrane components or accumulation of unusual constituents within the thylakoid membrane. Plastoglobuli are larger and more variable in size in these specimens than they are in most red algal chloroplasts. Large plastoglobuli commonly were located near the inclusions. The great electron density of plastoglobuli is due to the osmiophilic nature of the hydrophobic compounds of which they are composed (Kirk & Tilney-Bassett, 1978). Perhaps enrichment of thylakoids with similar compounds could transform

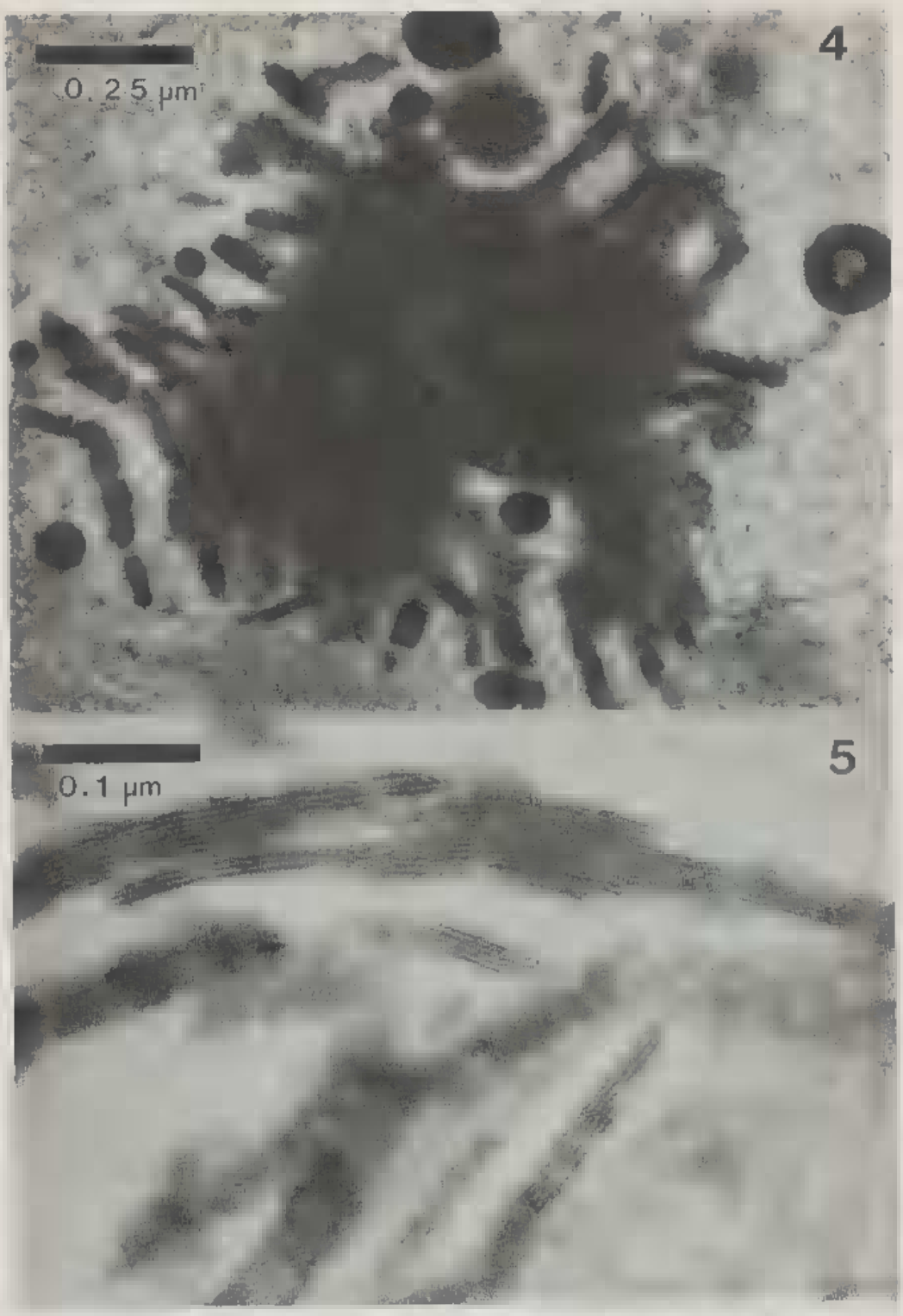

Fig. 4, 5 - Chloroplasts of *Trailliella* phase of *Bonnemaisonia hamifera*. Fig. 4. A cluster of inclusions is embedded in an amorphous, electron-dense matrix. Numerous plastoglobuli (P) associated with the inclusion. Fig. 5. High magnification of unstained section shows the alternating layers of electron-dense and electron-transparent material that make up the inclusions.

thylakoids into layered inclusions. However, until the inclusions are isolated and characterized, their origin and specific chemical nature will remain speculative.

Bonnemaisonia and other members of the Bonnemaisoniales are known to be involved in halogen metabolism (e.g. Codomier *et al.*, 1983; Fennical, 1975; Wolk, 1968), so the possibility that these unusual chloroplast inclusions may be related to this activity was considered, but X-ray microanalysis failed to detect halogens in the inclusions. Furthermore, halogen-containing compounds ▬ typically accumulated in specialized lateral cells, vesicle cells (Codomier *et al.*, 1983; Wolk, 1968), rather than the unspecialized axial cells, which contained the plastid inclusions.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to J. Seraji-Bozorgzad, H. Eichelberger, and H.N. Trick for technical assistance and Dr. Olivier Martin for assistance with translation.

REFERENCES

CODOMIER L., CHEVALIER S., JUPIN H., FRANCISCO C. & BANAIGS B., 1983 - Rhodophycées à iodures et Rhodophycées à bromures. Etude réalisée à la microsonde électronique. *Biol. Cell* 48: 75-80.

DELIVOPOULOS S.G. & KUGRENS P., 1985 - Thylakoid formation from coiled lamellar bodies during carposporogenesis in *Faucheocolax attenuata* Setch. (Rhodophyta, Rhodymeniales). *J. Cell Sci.* 75: 215-224.

FENICAL W., 1975 - Halogenation in the *Rhodophyta*. *J. Phycol.* 11: 245-259.

GOFF L.J., 1982 - The biology of parasitic red algae. *Progr. Phycol. Res. Progr.* 1: 289-369.

KIRK J.T.O. & TILNEY-BASSETT R.A.E., 1978 - *The Plastids*. 2nd Ed. Ed. New York, Elsevier/North Holland Biomedical Press, 960 p.

PUESCHEL C.M., 1988 - Cell sloughing and chloroplast inclusions in *Hildenbrandia rubra* (Rhodophyta, Hildenbrandiales). *Brit. Phycol. J.* 23: 17-23.

PUESCHEL C.M., 1990 - Cell structure. *In* COLE K.M. & SHEATH R.G. (Eds), *Biology of the Red Algae*. New York, Cambridge University Press, pp. 7-41.

PUESCHEL C.M., 1992 - An ultrastructural survey of the diversity of crystalline, proteinaceous inclusions in red algal cells. *Phycologia* 31: 489-499.

PUESCHEL C.M. & COLE K.M., 1980 - Phytoferritin in the red alga *Constantinea* (Cryptonemiales). *J. Ultrastruct. Res.* 73: 282-287.

PUESCHEL C.M. & PARTHASARATHY M.V., 1984 - X-ray microanalysis of phytoferritin in *Constantinea* (Cryptonemiales, Rhodophyta). *Phycologia* 23: 465-469.

PUESCHEL C.M. & VAN DER MEER J.P., 1984 - Ultrastructural characterization of a pigment mutant of the red alga *Palmaria palmata*. *Canad. J. Bot.* 62: 1101-1107.

WOLK C.P., 1968 - Role of bromine in the formation of the refractile inclusions of the vesicle cells of the Bonnemaisoniaceae (Rhodophyta). *Planta (Berl.)* 78: 371-378.

YOUNG D.N., 1979 - Ontogeny, histochemistry and the fine structure of cellular inclusions in vegetative cells of *Antithamnion defectum* (Ceramiaceae, Rhodophyta). *J. Phycol.* 15: 42-48.